Expression Induced by Interleukin-6 of Tissue-type Transglutaminase in Human Hepatoblastoma HepG2 Cells*

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We examined the effect of interleukin-6 (IL-6) on the expression of transglutaminase in human hepatoblastoma HepG2 cells. Treatment of cells with IL-6 increased their transglutaminase activity in a time- and dose-dependent way. Dexamethasone strengthened the stimulation by IL-6. Half-maximum stimulation of transglutaminase activity in the cells occurred at a dose of 40 pm IL-6 regardless of the presence of dexamethasone. Based on its immunoreactivity, the transglutaminase induced was identified as tissue-type transglutaminase. Immunoblot analysis showed that the increase in transglutaminase activity was related to an increase in the amount of transglutaminase protein. Northern blot analysis with a cDNA probe specific for human tissue-type transglutaminase showed that exposure of HepG2 cells to IL-6 increased the mRNA level of the enzyme, and the increase was detectable 3 h after IL-6 was added. Induction of the mRNA was maximum between 10 and 14 h. The increase in the mRNA level was not blocked by the presence of cycloheximide, suggesting that the increase was independent of protein synthesis. Injections into mice of substances that induce inflammation such as turpentine and lipopolysaccharides increased the liver transglutaminase activity. These results indicated that transglutaminase may be involved in some biological processes in hepatocytes regulated by IL-6 signaling.

Transglutaminases (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) are calcium-dependent acyltransferases that catalyze the formation of an amide bond between the γ-carboxyamide group of peptide-bound glutamine residues and the primary amine group of various amines, including the ε-amino group of lysine in certain proteins. These enzymes are widely distributed in tissues and fluids of animals and classified into at least four groups based on their biochemical properties: plasma, tissue, epidermal, and keratinocyte types. Several are involved in diverse biological functions in which they catalyze the formation of γ-(γ-glutamyl) lysine cross-links with protein substrates (for reviews, see Refs. 1–4). These functions include stabilization of fibrin structure in blood clotting (5) and in endogenously occurring fibrinolysis (6), formation of a cornified envelope inside epidermal keratinocytes (7), stiffening of the erythrocyte membrane (8), and wound healing (9). Transglutaminases also seem to be involved in the regulation of cellular growth and differentiation (10–13).

Liver transglutaminase is one of the most extensively studied tissue-type transglutaminases, but its physiological role is not clearly understood. Hand et al. (14) reported a reduction of cytosolic transglutaminase activity in chemically induced liver carcinogenesis. A peritoneal injection of retinoic acid (15) increased the activity of this enzyme in rat liver. Liver transglutaminase may participate in the apoptosis of hepatocytes (16, 17) and in the formation of cross-linked protein matrices at sites of cell-to-cell contact (18). Cytosolic transglutaminase activity of guinea pig liver increases during the postnatal growing phase, and this change may be involved in the postnatal development of liver cells (19).

Interleukin-6 (IL-6) is a multifunctional cytokine that helps to regulate immune responses, hematopoiesis, and inflammatory reactions (for review, see Ref. 20). It has also been called B cell stimulatory factor-2 (21), interferon β2 (22), hybridoma-plasmacytoma growth factor (23), myeloid blood cell differentiation-inducing protein (24), and hepatocyte-stimulating factor (25–27). The name "hepatocyte-stimulating factor" was given because of its role as a regulator of acute-phase protein synthesis and its secretion in hepatocytes (27). In human primary hepatocytes (28, 29), IL-6 induces the synthesis of fibrinogen, haptoglobin, serum amyloid A, C-reactive protein, and α1-antichymotrypsin, and represses the synthesis of albumin and transferrin; in the rat, IL-6 regulates the transcription of acute-phase protein genes in liver (30).

In rat hepatocytes, IL-6 stimulates the expression of the metallothionein (31) and T-kininogen (32) genes. IL-6 also represses DNA synthesis in rat primary hepatocytes (33). The distribution of labeled IL-6 after its injection into rats suggests that the liver is the major target organ of IL-6 (34). Rat hepatocytes have IL-6 receptors with a ligand-binding chain, which is structurally similar to that of human leukocytes (35).

These findings indicated that IL-6 is a signaling factor for various functions of liver cells and led us to investigate whether transglutaminase may be involved in known and unknown biological processes in hepatocytes regulated by the action of the multifunctional cytokine IL-6. For these studies, we used HepG2 cells, a liver cell line derived from a human hepatoblastoma. This cell line expresses a variety of liver-specific metabolic functions, so it should be useful experimentally (36). HepG2 cells have two kinds of IL-6 binding sites with low and high affinity; when the cells are treated with IL-6, the expression of acute-phase protein genes is stimulated (37). In this paper, we report that IL-6 induces the expression of tissue-type transglutaminase in HepG2 cells.

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1 The abbreviations used are: IL-6, interleukin-6; kb, kilobase; kbp, kilobase pair.
EXPERIMENTAL PROCEDURES

Cell Culture—A human hepatoblastoma cell line, HepG2, was obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in minimum essential medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37°C in a humid atmosphere of 95% air and 5% CO2. The effects of IL-6 (recombinant human IL-6, a gift from Ajinomoto Co., Inc., Japan) were tested by the addition of IL-6 to the medium after initial incubation for 24 h. Culture was started at the density of 2 x 104 cells in dishes with 6-cm diameters and containing 5 ml of medium. Endotoxin was not detected in the medium used.

The analysis was done by using the Immoblot assay kit, Toxicolor System (Seikagaku Corporation, Tokyo).

Animals—Male C3H/Hej mice (10 weeks old) were kept at 24°C in a cycle of 12 h of light and 12 h of darkness and fed ad libitum with a standard chow obtained commercially. The mice were injected intraperitoneally with lipopolysaccharide (10 µg dissolved in 300 µl of phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl)) or subcutaneously with turpentine (50 µl). Twenty-four hours after the injection, the mice were killed and the liver was removed and assayed for transglutaminase.

Transglutaminase Assay—Cells grown in culture dishes were harvested and washed in ice-cold phosphate-buffered saline containing 1 mM EDTA. The washed cells were suspended in 0.2 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 1 mM (p-amidinophenyl)-methanesulfonyl fluoride, and 0.2 mM dithiothreitol) and disrupted by five cycles of freezing and thawing. The cell lysate was layered on the cytosol and particulate fractions by centrifugation at 100,000 g for 1 h. The supernatant was collected as the cytosol fraction. The precipitate, after being washed twice by suspension in 2 ml of lysis buffer and centrifugation at 100,000 g for 1 h each time, was resuspended in an appropriate volume of the lysis buffer and used as the particulate fraction. The cell lysates were assayed for transglutaminase, giving the total transglutaminase activity, and the cytosol and particulate fractions were assayed, giving the activity of each fraction. Blood was removed from mouse livers by their perfusion with ice-cold 0.9% NaCl, after which the livers were cut into small pieces with scissors. The pieces of liver were made into 20% (w/v) homogenates with a motor-driven Teflon homogenizer. The homogenate was assayed, giving the transglutaminase activity of the liver. Transglutaminase activity was measured by the filter paper method (38) with acetyl α-casein (19) and [2,5-3H]histamine (4) as the substrates. The assay mixture contained, in a total volume of 100 µl, 50 mM Tris-HCl, pH 7.5, 5 mM CaCl2, 20 mM dithiothreitol, 4 µg/ml acetyl α-casein, 1 mM [5,5-3H]histamine (4 µCi), and an enzyme sample. The reaction was started by addition of the assay system, indicating that the transglutaminase activity detected seemed not to be a zymogenic transglutaminase like blood coagulation factor XIII, which is activated by incubation of the lysates at 37°C for 1 h or by treatment with thrombin (data not shown). Therefore, the transglutaminase activity detected seemed not to be a zymogenic transglutaminase like blood coagulation factor XIII, which is activated through limited proteolysis by some proteases.

When the culture of HepG2 cells was treated with 0.8 nM IL-6 for 24 h, the total transglutaminase activity increased 3-fold (Fig. 1). The activity was increased rapidly after incubation.

RESULTS

Transglutaminase activity (8.7 units/mg of protein) was found in HepG2 cell lysates. The activity was inhibited completely by the addition of 80 mM EDTA or 20 mM iodoacetamide to the assay system, indicating that the transglutaminase found in HepG2 cells is calcium-dependent and has SH group(s) essential for its catalytic function, like most animal transglutaminases examined (1, 2). The transglutaminase activity in the HepG2 cell lysates was not affected by incubation of the lysates at 37°C for 1 h or by treatment with thrombin (data not shown). Therefore, the transglutaminase activity detected seemed not to be a zymogenic transglutaminase like blood coagulation factor XIII, which is activated through limited proteolysis by some proteases.

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Immunoblot Analysis—As described by Laemmli (40), cell lysates were made soluble in a sample buffer, and cell proteins were separated during SDS-9% polyacrylamide gel electrophoresis. Immunoblot analysis was done essentially by the method of Burnette (41), and details were described before (42). Human plasma transglutaminase (factor XIII) was purchased from Calbiochem. The reaction between antigen proteins and rabbit polyclonal antibodies to guinea pig liver transglutaminase was detected with peroxidase-labeled secondary antibodies to rabbit immunoglobulin G by a chemiluminescence reaction with an ECL-Western blotting detection system (Amersham International) using the procedure described by the supplier. A 1.0-kbp fragment (nucleotide positions 600-1600) of human tissue transglutaminase cDNA was prepared from a plasmid containing the cDNA of human endothelial cell transglutaminase (45) (the gift of Drs. V. Gentile and P. J. A. Davies, University of Texas Medical School, Houston). A 1.1-kbp fragment of actin cDNA was obtained from a plasmid containing mouse actin cDNA (pAL41) (46) by PstI digestion. The size markers used were those in the RNA ladder kit (Life Technologies, Inc., Gaithersburg, MD).

FIG. 1. Stimulation of transglutaminase activity in HepG2 cells by IL-6. Cells were plated as described in the text and incubated in the presence of: O, no additive; ●, 0.8 nM IL-6; ▲, 1 µM dexamethasone; or ■, 0.8 nM IL-6 and 1 µM dexamethasone. At different times after the addition, total transglutaminase activity in the cells was assayed. Activities were calculated as per milligram of total cellular protein and shown as relative to the activity of the untreated control cells (8.7 units/mg protein) at zero time of incubation.
tion for 8 h and reached near its maximum by 16 h. Activity remained high for at least 8 h. Dexamethasone at the concentration of 1 mM increased the stimulatory effect of IL-6 almost 2-fold. Little change in the transglutaminase activity was found in the cells treated with 1 mM dexamethasone alone or in untreated control cells incubated for 24 h. The increase in transglutaminase activity depended on the concentration of IL-6 added in both the presence and the absence of dexamethasone (Fig. 2). Regardless of the presence of dexamethasone, half-maximum stimulation occurred when IL-6 was present at the concentration of about 40 pM, and this concentration was on the order of the dissociation constant, 15 pM, for the high affinity IL-6 binding sites of HepG2 cells (37). Stimulation was maximum between 0.6 and 1.5 nM IL-6.

Immunoblot analysis was done of lysates of HepG2 cells treated with IL-6 to identify any changes in the amount of transglutaminase during culture. Polyclonal antibodies to guinea pig liver transglutaminase that did not form antibody-antigen complexes with human factor XIII showed that HepG2 cells contained tissue-type transglutaminase with a molecular mass of 80 kDa (Fig. 3, lanes 1–3). The amount of 80-kDa transglutaminase protein increased with the time of IL-6 treatment (Fig. 3, lanes 3–6), but the amount in untreated control cells was unchanged after 18 h of incubation (Fig. 3, lane 7). Dexamethasone strengthened the induction by IL-6 (Fig. 3, lane 10) by about the same extent as it increased enzyme activity.

In HepG2 cells, about 90% of the total transglutaminase activity was in the cytosol fraction and the other 10% of the activity was in the particulate fraction. The treatment of HepG2 cells with IL-6 stimulated the increase in transglutaminase activity in both fractions at a similar rate (Table I).

The kinetics of the accumulation of tissue-type transglutaminase mRNA in HepG2 cells treated with IL-6 is shown in Fig. 4. A single 4.0-kb band (tissue-type transglutaminase mRNA) had increased significantly 3 h after the addition of IL-6, and a maximum was reached at about 14 h. Dexamethasone strengthened the induction by IL-6 of transglutaminase mRNA in HepG2 cells treated with IL-6 (Fig. 4, lane 10) by about the same extent as it increased enzyme activity.

FIG. 2. Dose response to IL-6 by change in transglutaminase activity in HepG2 cells. Cells were plated as described in the text and treated with IL-6 (C) or IL-6 plus 1 mM dexamethasone (O) at the concentrations indicated for 18 h. Total transglutaminase activity in the cells was assayed. Activities were calculated as per milligram of total protein and shown as relative to the activity of the control cells not treated with IL-6 (8.6 units/mg protein).

Injections of turpentine or lipopolysaccharide, often used to induce the acute-phase response in experimental animals, increased the liver transglutaminase activity in mice (Table II). Induction of the acute-phase response by turpentine or lipopolysaccharide was confirmed by immunochemical detection of an increase in the amount of haptoglobin, a typical acute-phase protein, in plasma from the mice (data not shown). IL-6 (0.8 nM) increased the transglutaminase activity of human hepatoma Hep3B cells 6-fold during culture for 18 h.

DISCUSSION

Our results as reported here suggest a relationship between IL-6 signaling and the induction of transglutaminase in the hepatocytes of mammals. Perhaps tissue-type transglutaminase is involved in the acute-phase response of hepat-
Interleukin-6-induced Expression of Transglutaminase

An acute-phase response was induced in mice by an injection of turpentine (50 μl) or lipopolysaccharide (10 μg). At 24 h after the injection, the mice were killed, the liver was removed and homogenized, and transglutaminase activity in the homogenate was assayed as described in the text. Means ± standard deviation are shown (n = 6).

<table>
<thead>
<tr>
<th>Injection</th>
<th>Transglutaminase activity (units/mg protein)</th>
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<tbody>
<tr>
<td>None</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Turpentine</td>
<td>7.4 ± 1.1</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>13.0 ± 1.1</td>
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IL-6 induces the differentiation of B and T cells, macrophages, and neural cells and also the growth of hematopoietic stem cells and mesangial cells. IL-6 increased 7-fold the transglutaminase activity of cultured mouse myeloid leukemia M1 cells, which are made to differentiate into mature macrophage-like cells by IL-6 (48). It is not known if there is a relationship between IL-6 signaling and the differentiation of liver cells. Perhaps transglutaminase participates in the differentiation induced by IL-6 of several kinds of cells.

The increase in transglutaminase activity in HepG2 cells treated with IL-6 seemed to be related to the increased synthesis of this enzyme and to the increased level of the mRNA for tissue-type transglutaminase. The IL-6-induced increase in the mRNA level seemed to be independent of protein synthesis, but it is not clear whether IL-6 increased the stability of the mRNA or increased the relative transcription rate of the transglutaminase gene. Rat and human genes of various acute-phase proteins, the synthesis and secretion of which are induced by IL-6, contain IL-6-responsive elements in their 5'-upstream regions, and the induction by IL-6 of these proteins is probably caused by an increase in the transcription rate of their genes (20, 27). The IL-6-responsive elements of several acute-phase genes such as those coding for hemopexin, haptoglobin, C-reactive protein, and α1-acid glycoprotein have a consensus sequence recognized by the transcription-controlling factor NF-IL-6, a nuclear factor for IL-6 expression (49). The IL-6-induced transcription of these genes seems to be regulated at least in part by this factor. Preliminary studies indicated that the 5'-upstream region of the guinea pig gene for liver transglutaminase contains the consensus sequence of the NF-IL-6 recognition sites. If these findings are taken into account, the IL-6-induced increase in the transglutaminase mRNA level in HepG2 cells is likely to be caused through an increase in the relative transcription rate of the corresponding gene.

The increase by cycloheximide of the level of transglutaminase mRNA might occur because this mRNA is subject to transcriptional regulation that is under the control of labile repressor proteins. Alternatively, cycloheximide might prolong the biological half-life of transglutaminase mRNA as the mRNAs of histones H3 and H4 are stabilized by protein synthesis inhibitors (50). Superinduction of transglutaminase mRNA in the presence of cycloheximide has been observed in cultured human erythroleukemia cells (51). Cycloheximide had no effect on the level of tissue-type transglutaminase mRNA in mouse resident peritoneal macrophages (52). These findings suggest that the expression of tissue-type transglutaminase genes in various cells may or may not be dependent on the protein synthesis, depending on the kind of cells.

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\(^2\) N. Suto, K. Ikura, and R. Sasaki, unpublished observations.

\(^3\) N. Suto, K. Ikura, R. Shinagawa, and R. Sasaki, unpublished observations.
Treatment of HepG2 cells with the synthetic glucocorticoid dexamethasone leads to up-regulation of their IL-6 receptors (53). This probably explains our finding that dexamethasone treatment alone did not increase the mRNA and protein levels of transglutaminase in HepG2 cells but that dexamethasone strengthened their induction by IL-6. Monokines such as IL-1, IL-6, and tumor necrosis factor that are secreted from monocytes and macrophages during inflammation stimulate the release of adenocorticotropic hormone from pituitary cells, increasing the secretion of glucocorticoids by adrenocortical cells. The glucocorticoids may help to regulate the synthesis of tissue-type transglutaminase as well as the synthesis of acute-phase proteins in hepatocytes.

To our knowledge, this is the first report describing the induction of tissue-type transglutaminase by IL-6. Several other factors induce tissue-type transglutaminase in different cell systems. Retinoic acid increases the level of tissue-type transglutaminase in mouse macrophages (52) and human erythroleukemia cells (51) by increasing the rate of gene transcription. Morphological changes caused by retinoids in vascular endothelial cells are associated with an increase in tissue-type transglutaminase and its mRNA level (54, 55). Transforming growth factor-β induces tissue-type transglutaminase in differentiated normal human epidermal cells but does not induce their terminal differentiation (56). Sodium butyrate can also induce the activity and expression of tissue-type transglutaminase in cultured rat pheochromocytoma PC12 cells (57) and virus-transformed human embryonic fibroblasts (58). These findings suggest that as with other eucaryotic genes, the expression of tissue-type transglutaminase can be controlled by various signaling factors so that the enzyme works effectively and when needed in different tissues.

REFERENCES